Impacts of the human pharmaceutical diclofenac in the aquatic environment

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To the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences, October 2009

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been published and approved for the award of a degree by this or any other University.

Signature ...A. MEHINTO....

Abstract

An increasing number of pharmaceuticals have been found in the aquatic environment and the issue has become a human and environmental health concern. Many pharmaceuticals are not fully degraded in wastewater treatment plants (WWTPs) and are continuously released in the aquatic environment resulting in concentrations in the low µg/l range in the receiving waters. Diclofenac is a widely used non-steroidal anti-inflammatory drug (NSAID) and is persistent in the aquatic environment. This pharmaceutical has been frequently reported in wastewater effluents, surface waters, groundwaters and even drinking water. NSAIDs are known to inhibit the cyclooxygenase activity, an enzyme present in many species of the animal kingdom responsible for the synthesis of prostanoids, and chronic exposure to environmental diclofenac may have detrimental effects on metabolism of non-target organisms including microbes and fish. In this thesis, microbiology, genomics and metabolomics approaches were used to investigate the effects of diclofenac on aquatic microbes and fish.

In the first study of the thesis (chapter 3), the biodegradation of selected NSAIDs was investigated, together with their potential toxicity to aquatic microbes. Aerobic biodegradation experiments were conducted using activated sludge and wastewater effluents as microbial inocula and diclofenac, ketoprofen or naproxen as sole carbon source (1-10 mg/l) in order to isolate and identify the bacterial degraders. Changes in the bacterial populations were monitored by optical density and PCR-DGGE. The analytical techniques solid phase extraction (SPE) and ultraperformance liquid chromatography-mass spectrometry (UPLC-TOF-MS) were optimised to quantify the pharmaceuticals in environmental samples. High recovery rates were obtained with 94% for diclofenac; 92% for ketoprofen and 85% for naproxen and with detection capabilities down to 3-7 ng/l. Results from the biodegradation experiments showed that ketoprofen and naproxen were eliminated at up to 99 and 55% respectively over a 40 days period. Consistently with previous studies, diclofenac showed no significant degradation. In all the enrichments, a significant decrease in the bacterial abundance was observed as a consequence of NSAIDs exposure and attempts to isolate the bacterial degrading populations were unsuccessful. Given the apparent micro-toxicity of these NSAIDs, the standardised test Microtox$^{@}$ was carried out with *Vibrio fischeri*. The EC_{50} (15 min) estimated ranged from 13.5 mg/l $\pm$ 2.3 for diclofenac to 42.1 mg/l $\pm$ 3.9 for naproxen. Further toxicological tests were performed with diclofenac on bacterial strains isolated from activated sludge. Growth inhibitory effects were observed from 50-70 mg/l for *Micrococcus luteus*, *Zoogloea ramigera and Comamonas denitrificans*. *Pseudomonas putida* seemed more tolerant to diclofenac exposure and toxic effects were observed from 90 mg/l. These studies showed that diclofenac was the most toxic NSAID but toxicological effects in bacteria only occurred at concentrations at least 1,000 times higher than those found in the environment. However, chronic exposure to lower concentrations may cause similar interferences and affect the degradation potential of naturally occurring microbial populations.

The second study (chapter 4) investigated the biological effects of sub-chronic exposure to waterborne diclofenac (0.5, 1, 5 and 25 µg/l) in female juvenile rainbow trout *Oncorhynchus mykiss*. After 21-day exposure, mRNA expression levels of cytochrome p450 1a1 (*cyp1a1*), cyclooxygenase (*cox*) 1 and 2, and *p53* were investigated in the liver, kidney and gills using RT-PCR and QPCR. These genes were selected as they are likely targets for diclofenac in mammals. Histopathological investigations were carried out in the small intestine, liver and kidney because

diclofenac has been reported to induce toxicity responses in these tissues. Fish bile was also analysed by SPE and UPLC-TOF-MS to evaluate the bioconcentration potential of diclofenac and look for evidences of diclofenac metabolism. Results showed a significant reduction of both *cox1* and *cox2* expression in the liver, gills and kidney from 1 µg diclofenac/l. In contrast diclofenac induced an increase in mRNA levels for *cyp1a1* in the liver and gills but a significant reduction of *cyp1a1* expression in the kidney from 1 µg/l. There were no clear effects of diclofenac on the mRNA levels of *p53*. Diclofenac exposure caused tissue damages at exposure concentrations as low as 1 µg/l. Histopathological injuries included inflammation, hyperplasia and fusion of the villi in the small intestine and tubule necrosis in the kidney. There were no obvious changes in the liver of diclofenac-exposed fish. The analysis of bile revealed a bioconcentration potential between 509 $\pm$ 27 and 657 $\pm$ 25. A reactive metabolite of diclofenac was also detected at the highest exposure concentration which may be responsible for the severe injuries found in those fish. Sub-chronic exposure to environmental concentrations of diclofenac altered gene expression and it is possible that long term exposure to environmental diclofenac lead to significant impacts on fish health.

In the final part of this thesis (chapters 5 and 6) effects on the metabolite composition of biofluids were analysed in diclofenac-exposed fish. This work entailed developing and validating appropriate methodologies to analyse fish bile and blood plasma. Methanol extraction and UPLC-TOF-MS were optimised to analyse the plasma metabolome but the methodologies were not suitable to detect low abundance molecules such as eicosanoids due to the interferences (ion suppression) in the samples matrix. Multivariate data analysis failed to detect the endogenous metabolites of the plasma affected by the chemical exposure. The only discriminating metabolite was found after analysis of the plasma samples from control vs. 25 µg/l treatment groups and identified as the exogenous compound diclofenac. To analyse the bile, the developed SPE methodology was carried out in order to separate the metabolites between a free steroids (fatty acids, eicosanoids, etc.) fraction and a conjugated steroids (bile salts) fraction. Due to high levels of taurocholic acid masking other metabolites in the conjugated fraction, some bile samples were hydrolysed to deconjugate these metabolites. The non-hydrolysed and hydrolysed bile fractions were analysed by UPLC-TOF-MS in positive and negative ionization. Multivariate data analysis using principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) revealed significant perturbations in the bile metabolite profile of diclofenac-exposed rainbow from the lowest exposure concentration (0.5 µg/l). Over 50 metabolites were elevated or reduced as a result of the 21-day exposure, suggesting that diclofenac affected several metabolic pathways. One metabolite was identified as a lipooxygenase product. This suggests that the inhibition of prostanoids synthesis can cause a shift in the arachidonic cascade and increase the synthesis of other eicosanoids. Most of the other discriminative metabolites remain unidentified and FT-MS analysis will be performed to obtain a structural identity. The metabolomics study further highlights the concern of environmental diclofenac in non-target organisms and the need to investigate the metabolic pathways affected.

Acknowledgements

First of all I would like to thank my supervisors Professor Charles Tyler, Dr Sara Burton and Professor Hilary Lappin-Scott for their support and encouragement throughout this study. I am eternally grateful to my unofficial supervisor Dr Elizabeth Hill from University of Sussex for teaching me metabolomics and for her invaluable help with the chemical analyses.

Special thanks to Peter Splatt and Jan Shears for the vast technical support. I would like to thank Morley Williams from Southwest Water from (Countess Wear) for its help during sampling. I would also like to thank Dr Eduarda Santos, Dr Amy Filby, Dr Patrick Hamilton, Dr Lisa Bickley, and Dr Anke Lange for their precious help in the lab and during the writing process. I am bery grateful to all the members of the former EMERGE group and the EMFB group at University of Exeter and my colleagues at University of Sussex for their friendship and support. Special mentions must be made to Viv, Rhys, Tess, Jenny and Luanne who helped me during the fish exposure but also Rachel, Max, Theresa, Laura and Okhyun. Big thank you to Dalia and Nicola for their precious advice to "carry on regardless" and support. A huge thank you to Elena for guiding me through the metabolomics, and being such a good friend.

Thank you to Marco, Hervé, tante Colette, tante Arlette, my family and friends who have supported me during the PhD. Special thank you to my cousin Dr Bobo for everything…

Finally a huge thank you to my parents for their unconditional love, patience, support and believing in me.

This thesis is dedicated to my aunts Madina Kelani (1952-2009), Yvette Paraiso (1963-2008) and my cousin Titi Bouraima (1969-2008).

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List of abbreviations

2D-PAGE	2-dimensional polyacrylamide gel electrophoresis
AHH	aryl hydrocarbon hydroxylase
ANOVA	analysis of variance
BLAST	Basic Local Alignment Search Tool
BC	bioconcentration
bp	base pair
BPI	base peak intensity
CAS	chemical abstract service
cDNA	complementary deoxyribonucleic acid
CE-MS	capillary electrophoresis - mass spectrometry
cm	centimetre
cox	cyclooxygenase
C_T	threshold cycle
cyp	cytochrome P450
cyp1a1	cytochrome P450 1a
Da	dalton
dATP	deoxyadenosine triphosphate
DCF	diclofenac
dCTP	deoxycytidine
dGTP	deoxyguanosine triphosphate
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
dNTPs	deoxyribonucleotide triphosphates
dTTP	thymidine triphosphate
E_2	17β-estradiol
EC_{50}	50% effect concentration
ECOSAR	Ecotoxicological Structure Activity Relationship
EDC	endocrine disrupting chemical
EE_2	17α-ethinylestradiol
EPA	eicosapentaenoic acid
EROD	ethoxyresorufin-*O*-deethylase
ESI	electrospray ionisation
eV	electron volt
FT-MS	Fourrier transform - mass spectrometry
g	gram
GC	gas chromatography
HEPES	hydroxyeicosapentaenoic acids
HETES	hydroxyeicosatetraenoic acids
HPLC	high performance liquid chromatography
hr	hour
IMS	industrial methylated spirit

IS	internal standard
Kg	kilogram
kV	kilo volt
l	litre
LC	liquid chromatography
LOD	limit of detection
LOEC	lowest observed effect concentration
M	mole per litre
M+H	protonated molecule
M-H	deprotonated molecule
MEC	measured environmental concentration
mg	milligram
MIC	minimum inhibitory concentration
min	minute
ml	millilitre
mm	millimetre
mM	millimole per litre
M-MLV	moloney murine leukaemia virus
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MSM	minimal salts medium
MW	molecular weight
m/z	mass to charge ratio
n	number of samples
ng	nanogram
nm	nanometre
nM	nanomole per litre
NMR	nuclear magnetic resonance
NSAIDs	non steroidal anti-inflammatory drugs
OD	optical density
OPLS	orthogonal partial least squares projection to latent structures
p	statistical probability
P450	cytochrome P450
p53	tumour protein 53
PAH	polycyclic aromatic hydrocarbon
PCA	principal component analysis
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
PEC	predicted environmental concentration
pg	picogram
PIT	passive integrated transponder
PLS-DA	partial least squares projection to latent structures discriminant analysis
pmol	picomole
PNEC	predicted no effect concentration

ppm	part per million
Q^2	cumulative variation predicted by the PCA or PLS model
Q-PCR	real-time quantitative reverse transcription PCR
R^2	variation explained by the PCA or PLS model
RNA	ribonucleic acid
Rnase	ribonuclease
rpl8	ribosomal protein l8
rpm	rotation per minute
rRNA	ribosomal ribonucleic acid
RT	retention time
RT-PCR	reverse transcription polymerase chain reaction
SE	standard error
sec	seconds
SPE	solid phase extraction
TIC	total ion chromatogram
T_M	melting temperature
TOF	time of flight
UPLC-MS	ultraperformance-liquid chromatography - mass spectrometry
UV	ultraviolet
v/v	volume to volume ratio
V	volt
vs.	versus
WWTP	wastewater treatment plant
°C	degree Celsius
µg	microgram
µl	microlitre
µm	micrometre
µM	micromole per litre

List of abbreviated chemicals

11-HETE	±11-hydroxyeicosatetraenoic acid
$CoCl_2$	phosgene
CTAB	cetyltrimethylammonium bromide
$CuSO_4$	copper (II) sulphate
E_2-*d4*-S	[2,4,16,16-d4] 17β-estradiol sodium 3-sulfate
EDTA	ethylenedinitrilotetraacetic acid
H_2SO_4	sulphuric acid
K_2HPO_4	potassium phosphate dibasic
KH_2PO_4	potassium dihydrogen phosphate
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
$MnSO_4$	manganese sulphate
NaCl	sodium chloride
NaOH	sodium hydroxide
Na_2SO_4	sodium sulphate
NH_4OH	ammonium hydroxide
$(NH_4)_2\ SO_4$	ammonium sulphate
P-*d9*	[2,2,4,6,6,17α-21,21,21-d9] progesterone
PGB_2	prostaglandin B2
PGE_2	prostaglandin E2
PGJ_2	prostaglandin J2
TAE	Tris-acetate-EDTA
TRIS	2-Amino-2-hydroxymethyl-propane-1,3-diol
TxB_2	tromboxane B_2
$ZnSO_4$	zinc sulphate